Original

Periodontal Tissue Formation by Reaggregation System in Mice

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Abstract: The reaggregation system is a special technique developed to clarify the essential characteristics of the mesenchyme. In this study, the reaggregation system was performed using mouse lower first molar at E13.5 and the reaggregated tooth germs were transplanted into the kidney capsule for 6 weeks to investigate tooth root formation. The reaggregated tooth was surrounded by bone. The reaggregated tooth germ produced a single thick root like a taurodontium (cuneiform tooth), although mouse lower first molar has two roots *in vivo*. Histologically, the reaggregated mesenchyme could form not only tooth elements including enamel, dentin, dental pulp and cementum, but also periodontal tissue including periodontal ligament-like structure and alveolar bone-like structure. The periodontal ligament-like structure has a regular width between the cementum and alveolar bone-like structure. Moreover, Sharpy's fiber-like structure was inserted into the cementum and alveolar bone-like structure. In conclusion, the reaggregated mesenchyme has the ability to form both tooth and periodontal tissue without information from the epithelium. In addition, this reaggregation system may be a useful method for regenerative dentistry, since tooth and periodontal tissue can be produced *en bloc*.

Key words: Periondontium, Reaggregation system, Molar, Mouse

Introduction

It is well known that tooth morphogenesis is an excellent model system for epithelial-mesenchymal interactions, which induce the initiation and development of tooth germs^{1, 2)}. These reciprocal tissue interactions lead to the position of individual tooth germ, cuspal patterning, cell differentiation and synthesis of mineralized matrices³⁾. In mouse first molar, the oral epithelium derived from ectoderm thickens in the putative tooth forming area on embryonic day 11 (E11). Next, the epithelium invaginates into the underlying mesenchyme derived from neural crest and forms the tooth bud on E12.5. At this stage, mesenchymal cells condense around the tooth bud. Then, the epithelial area of tooth germ, enamel organ, folds and creates a cap-like structure on E14. The mesenchymal cells condense at a hollow place under the enamel organ resulting in dental papilla. Moreover, enamel organ and dental papilla are covered by fibrous-rich mesenchyme, dental follicle, at this stage.

Previous recombinant experiments have demonstrated that the epithelium could induce tooth formation prior to the bud stage. However, the potential for tooth formation shifted from the epithelium to the mesenchyme at the early bud stage. At the late bud stage, the mesenchyme already takes the initiative for tooth formation against the epithelium⁴⁻⁶⁾.

The reaggregation system has previously already been used to examine a feather bud, a limb bud⁷⁾ and a tooth⁸⁾. Using the reaggregation system, the essential characterization of the mesenchyme cells as well as the effects of the epithelial-mesenchymal interaction can be examined. Previous reaggregation experiment using E13.5 mouse lower first molar tooth germ was demonstrated that the dental mesenchyme has the ability to produce a tooth crown, although the mesenchyme loses information from the dental epithelium as an essential characteristic. In addition, the newly formed tooth has an enamel structure similar to that of normal tooth *in vivo*⁸⁾. Since previous study only investigated formation up to 14 days after reaggregation, there is no information about tooth root formation of reaggregated tooth germs.

This study adopted a reaggregation system to investigate dental

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mesenchymal cells as in the previous study⁸⁾. After reaggregation experiment, reaggregated tooth germs were transplanted into kidney capsule for 6 weeks. Scanning electron microscope (SEM) observation and light microscopy (LM) were examined to investigate reaggregated tooth root formation. The results show that the reaggregated tooth formed a tooth root. Moreover, periodontal tissue, namely, cementum, periodontal ligament-like structure and alveolar bone-like structure, were formed *en bloc*. These findings suggest that this reaggregation system may be a useful method for regenerative and developmental research.

Materials and Methods

Animal Care and Use Committee of Nihon University School of Dentistry at Matsudo approved procedures used in this study (No. 03-0018).

Animals

Twenty-four ICR mice (*Mus musculus*) on embryonic day 13.5 (E13.5) (vaginal plug=E0) were examined.

Reaggregation system and kidney transplantation

The reaggregation system was described previously⁸⁾. Briefly, the first molar tooth germs from the lower jaw were dissected on E13.5 under a stereoscopic microscope in phosphate buffered saline (PBS). The germs were incubated at room temperature in 1.2 U/ml dispase II (Roche Diagnostics, Germany) for 12.5 min and washed in Dulbecco's minimum essential medium (Bio Whittaker, USA) containing 10% fetal bovine serum (GIBCO, USA). After separation of the epithelium and the mesenchyme, the epithelium remained intact in the media. The mesenchyme was triturated into single cells by drawing them through pipettes. The dissociated cells were repelleted by centrifugation (6,500 rpm for 4 min) and were allowed to reaggregate for 1 h at 37 °C. Then, the epithelium was combined with the reaggregated mesenchyme for 1 day at 37 °C. After 1 day incubation, the reaggregated tooth germs were transplanted into kidney capsule for 6 weeks using male adult mice as hosts.

Tissue preparation

The host mice were perfused with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for SEM and LM. For SEM observation, the reaggregated tooth including bone was dissected from the kidney. In some specimens, bone was detached from the reaggregated tooth. After taking a photo using a stereoscopic microscope (SZX12, OLYMPUS, Japan), the specimens were embedded in polyester resin (No. 105, Marumoto Struers, Japan) and were polished. The polishing surface was etched by 0.5% hydrochloride solution for 30 seconds. After critical point drying and coating with platinum, specimens were observed by SEM (S-2700, Hitachi, Japan). For light microscopy, reaggregated tooth including bone between the kidney capsule and cortex were

Figure 1 (A) Bone surrounding the reaggregated tooth. The reaggregated tooth is surrounded by bone except for occlusal portion of the tooth crown. (B) Side view of the reaggregated tooth. The reaggregated tooth has a single thick root. At the apical portion of the root, the cementum develops well (arrowhead). (C) Occlusal view of the reaggregated tooth. The shape of the reaggregated tooth differs from that of normal lower first molar. (D) Side view of the second reaggregated tooth. (E) Occlusal view of the second reaggregated tooth. The second reaggregated tooth looks like a dwarfed tooth. bars: 500μm

dissected out and fixed with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) at 4 °C overnight. The specimens were embedded in paraffin by conventional methods. Sections 5 μm thick were mounted on APS-coated slides, then stained with hematoxylin-eosin (H-E).

Results

Stereoscopic microscope observation

To clarify the state of the reaggregated tooth in the kidney capsule 6 weeks after transplantation, the stereoscopic microscope observation was performed. Since bone surrounded the reaggregated tooth, the occlusal area of tooth crown was observed on side view (Fig. 1-A). The reaggregated tooth had a completed crown and a single thick root (Fig. 1-B). Moreover, the apical part of root was thick (Fig. 1-B arrowhead). On occlusal view, the shape of tooth crown was not similar to normal lower first molar (Fig. 1-C). Occasionally, another reaggregated tooth (second reaggregated tooth) was found by the reaggregation system (Fig. 1-D, E). This second reaggregated tooth looked like a dwarfed tooth, since the size was very small and the shape was totally different from that of either lower first or second molar *in vivo* (Fig. 1-D, E).

SEM observation

Figure 2. (A) SEM image of the reaggregated tooth with bone. (B) Higher magnification of the apical part of (A). Bone (b) exists at the middle and apical part of the reaggregated tooth. However, cementum (c) is thick at the apical part of the reaggregated tooth, although cementum is thin at the middle part of the reaggregated tooth. (C) H-E section of whole reaggregated tissue. (D) Higher magnification of the cervical portion of (C). (E) Higher magnification of the apical portion of (C). The reaggregated tissue includes thick keratinized epithelium (ke), enamel (enamel space: es), dentin (d), acellular (ac) and cellular cementum (cc), dental pulp (p), periodontal ligament-like structure (pl) and alveolar bone-like structure (ab). Sharpy's fiber-like structure inserts into both the acellular and cellular cementum and alveolar bone-like structure. The collagen bundles did not run regularly and alveolar bone-like structure appears similar to woven bone. us: unidentified space, bars: (A) 333 μ m, (B) 150 μ m (C) 250 μ m, (D) 20 μ m, (E) 50 μ m

To analyze the three-dimensional structure of the reaggregated tooth surrounded by bone, ground surface was examined by SEM. The reaggregated tooth had an enamel-free area similar to that of normal mouse molar (Fig. 2-A). The enamel structure was divided into three layers based on the running patterns of enamel prisms like a normal molar (data not shown). At the apical portion of reaggregated tooth root, thick cellular cementum was observed between dentin and bone (Fig. 2-B). However, cellular cementum was not detected between dentin and bone at the middle part of tooth root (Fig. 2-A).

LM observation using H-E staining

To observe the fine structure of the reaggregated tooth, sections

were stained by H-E. Between the kidney capsule and cortex, thick keratinized epithelium, enamel space, dentin, pulp, cementum (acellular and cellular), periodontal ligament-like structure and alveolar bone-like structures were observed (Fig. 2-C). Alveolar bone-like structure was comprised of woven bone and completely surrounded the reaggregated tooth. Bundles of collagen fibers located in the periodontal ligament-like structure were inserted into cementum (acellular and cellular) and alveolar bone-like structure. However, collagen bundles did not run regularly in periodontal ligament-like structure. Next to the enamel space, there was an unidentified space that is not found in normal tooth development (Fig. 2-C). Some cells were detected in this unidentified space (Fig. 2-D). Between cementum and alveolar

bone-like structure, periodontal ligament-like structure showed a constant width. However, in some sections, the width of periodontal ligament is very narrow at the apical portion of the reaggregated tooth (data not shown), although there was no ankylosis between the cementum and alveolar bone-like structure in any sections. There was active bone marrow including megakaryocytes and precursor cells of leukocyte (data not shown) in the alveolar bone-like structure (Fig. 2-C).

Discussion

The reaggregation system is a useful method of investigating the essential properties of mesenchyme^{7,8)}. Previous reaggregation experiment using E13.5 mouse lower first molar demonstrated that the dental mesenchyme has an ability to produce the tooth as an essential property, although even dental mesenchyme loses information from the dental epithelium⁸⁾. However, since previous study investigated only the newly formed tooth crown formation process, there is no information about the root formation process of the reaggregated tooth. Therefore, reaggregated tooth germs were incubated in kidney capsules for 6 weeks to investigate reaggregated tooth root formation. As a result, the reaggregated tooth could form a root that included acellular and cellular cementum, although the reaggregated tooth had only a single thick root. Moreover, periodontal ligament-like structure and alveolar bone-like structures were observed with reaggregated tooth. In other words, tooth and periodontal tissue could be produced *en bloc* by the reaggregation system. In addition, Nakao et al.⁹⁾ described that the transplanted tooth germ into the subrenal capsule using incisor tooth germ in mice was surrounded by alveolar bone-like structure and periodontal ligament-like structure. These findings suggest that reaggregated mesenchymal cells can differentiate into cementoblasts, fibroblasts and osteoblasts respectively similar to normal periodontium development from a dental follicle, although reaggregated mesenchymal cells lose the signals including positional information from the dental epithelium. In order for this method to be used for regenerative treatment in the future, remaining problems must be solved. For example, the alveolar bone-like structure was woven bone and collagen bundles did not run regularly in the periodontal ligament-like structure, since the reaggregated tooth grew in the kidney capsule without mechanical stress. In clinical dentistry, however, it is considered that regeneration of tooth and periodontal tissue “*en bloc*” would be a better method than the “individual” regeneration method of tooth and periodontal tissue respectively. Therefore, this reaggregation system may be a useful method for both regenerative and developmental research and clinical dentistry, since tooth and periodontal tissue can form *en bloc*.

The number and shape of the tooth root were different from that of normal root. Reaggregated tooth has a single thick root like taurodontism (cuneiform tooth)¹⁰⁾. It was considered that

taurodontism may be caused by a failure of the invagination of Hertwig's epithelial root sheath in a horizontal direction¹¹⁾. Moreover, it was reported that taurodontism is involved in ectodermal abnormality¹²⁾. According to these reports, a new epithelial-mesenchymal interaction may be acquired by the reaggregation system. Moreover, recently it is reported that transplanted tooth using normal tooth germ of mandibular first molar in mice has only one tooth root, although cementum distribution pattern is similar to normal molar *in vivo*¹³⁾. These results suggest that the microenvironment surrounding tooth germ may be an important factor for multi-root formation.

In the present study, a second reaggregated tooth was occasionally found in the kidney capsule, although a third reaggregated tooth was never observed. This second reaggregated tooth was similar to dwarfed tooth, since the size and shape were completely different from those of normal first and/or second lower tooth. Since the shape of the crown was modulated by the number of mesenchymal cells¹⁴⁾, the formation of the second reaggregated tooth may depend on the ratios of cell number in the epithelium and reaggregated mesenchyme.

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